American Fern Journal 85(4):375–394 (1995)

A Molecular Assessment of Relationships Among Cryptic Species of *Botrychium* Subgenus *Botrychium* (Ophioglossaceae)

WARREN D. HAUK
Department of Biology, University of North Carolina, Chapel Hill, NC 27599-3280

ABSTRACT.—Phylogenetic relationships among 20 currently recognized species of *Botrychium* subgenus *Botrychium* were investigated using nucleotide sequences from the plastid gene *rbcL*. Analysis of eight diploid species produced a single most parsimonious tree with three distinct clades. The lanceolatum clade (one species) was sister to all other species. The lunaria clade (two species) was sister to the simplex-campestre clade (five species) which contained two weakly supported subclades. Single populations of *B. lunaria* and *B. simplex* were more closely related to other species in the same geographic region than to populations of their own species from distant geographic regions. In a second analysis that included both diploid and polyploid species, eight of twelve polyploid taxa had *rbcL* sequences identical to those of known diploids, which suggested relatively recent origins for these eight polyploid species. Four polyploids, *B. ascendens*, *B. paradoxum*, *B. ×watertonense*, and *B. minganense*, did not associate with a diploid species, and therefore represented polyploid derivatives of diploid taxa that were not sampled and possibly are extinct. Associations of diploid and polyploid species as determined by *rbcL* analysis are consistent with morphological hypotheses of polyploid origins with the exception of *B. ascendens*, which did not possess the *rbcL* sequence of either of its putative diploid progenitors.

Species of *Botrychium* subgenus *Botrychium*, commonly called moonworts, represent the smallest, most inconspicuous, and most species-rich subgroup of the genus. Although the mountains of western North America harbor the greatest number of species, subgenus *Botrychium* includes species that are distributed throughout most of the world (Clausen, 1938). However, the ranges of individual species vary remarkably. The most common species, *Botrychium lunaria* (L.) Sw., grows on nearly every continent, whereas *B. gallicomontanum* Farrar & Johnson-Groh is known only from a single locality. For many moonwort species, current knowledge of their ranges may reflect more the extreme difficulty involved in finding the plants in the field rather than their true distributions (Wagner and Wagner, 1994).

A distinguishing feature of Ophioglossaceae, including subgenus *Botrychium*, is that the entire aerial portion of the plant is composed of a single leaf divided into a fertile segment and sterile segment (Bower, 1926; Bierhorst, 1971; Foster and Gifford, 1974). In general, a single leaf is produced per year, and it is typically small in size, ranging from 1–15 cm in height. The small size and simplicity of the leaf have limited the number of discrete characters available for the construction of classifications and made the systematics of subgenus *Botrychium* particularly problematic and controversial (Tryon and Tryon, 1982).

As circumscribed by Clausen (1938), subgenus *Botrychium* contained 6 distinct species (Table 1) and 15 varieties or subspecies. Over the last several

TABLE 1. Species of *Botrychium* subgenus *Botrychium*. Preliminary sectional assignments based on morphological and cytological studies of Wagner and Wagner (pers. comm.). The number after each species name indicates the documented ploidy level of the species (2x = diploid, 4x = tetraploid, 6x = hexaploid), with x = 45. Asterisks indicate species sampled in the present study.

Clausen (1938)	Wagner & Wagner (pers. comm.)	Ploidy level
	Section *Lanceolatum*	
B. lanceolatum (J. Gmel.) Angstr.	**B. lanceolatum*	2x
	Section *Lunaria*	
B. lunaria (L.) Sw.	**B. lunaria*	2x
	**B. crenulatum* W.H. Wagner	2x
	B. pallidum W.H. Wagner	2x
	**B. minganense* Vict.	4x
	**B. spathulatum* W.H. Wagner	4x
	**B. ascendens* W.H. Wagner	4x
	Section *Matricariifolium*	
B. matricariifolium A. Braun	**B. matricariifolium*	4x
B. boreale Milde	*B. boreale*	?
	**B. acuminatum* W.H. Wagner	4x
	**B. hesperium* (Maxon & Clausen) W.H. Wagner & Lellinger	4x
	**B. echo* W.H. Wagner	4x
	**B. pedunculosum* W.H. Wagner	4x
	**B. pinnatum* St. John	4x
	**B. pseudopinnatum* W.H. Wagner	6x
	Section *Simplex*	
B. simplex E. Hitchc.	**B. simplex*	2x
B. pumicola Coville	**B. pumicola*	2x
	**B. montanum* W.H. Wagner	2x
	B. mormo W.H. Wagner	2x
	B. gallicomontanum Farrar & Johnson-Groh	4x
	Sectional status undetermined	
	**B. campestre* W.H. Wagner & Farrar	2x
	**B. lineare* W.H. Wagner	?
	**B. paradoxum* W.H. Wagner	4x
	**B.* ×*watertonense* W.H. Wagner	4x

decades the number of species has increased to 24 as a result of the detailed morphological and cytological studies of W. H. and F. S. Wagner (Table 1). Wagner and Wagner and other workers have used increasingly subtle morphological characters initially to recognize new species, many of which are polyploid (Wagner and Wagner, 1981, 1983b, 1986, 1990a, 1990b; Wagner et. al, 1984; Farrar and Johnson-Groh, 1991). Through the use of comparative field techniques (Wagner and Wagner, 1983a), environmentally stable and presumably genetically fixed morphological characters have been identified and correlated with cytological observations to yield current concepts of species delimitations (Wagner, 1993).

One major consequence of the morphological simplicity characteristic of subgenus *Botrychium* is that many species are extraordinarily difficult to distinguish. Because the morphological characters necessary to distinguish species are subtle, many species of subgenus *Botrychium* are characterized as "cryptic" species. Cryptic species were defined by Stebbins (1950) as, ". . . population systems which were believed to belong to the same species until genetic evidence showed the existence of isolating mechanisms separating them." According to Paris et al. (1989), cryptic species typically: 1) are poorly differentiated morphologically, 2) represent distinct evolutionary lineages because they are reproductively isolated, and 3) have historically been misinterpreted as members of a single species. Many species of subgenus *Botrychium* meet these criteria. First, they are extremely difficult to differentiate without considerable field experience. Small (<2cm) or immature individuals can be nearly impossible to identify for even the most experienced collectors. Second, the existence of sterile, putative hybrids with intermediate morphology and abortive spores has provided evidence of hybridization and reproductive isolation between species (Wagner, 1980, 1991; Wagner and Wagner, 1988; Wagner et al., 1985). Third, the majority of newly recognized species was separated from within a group previously recognized as a single species. For example, *B. pinnatum* H. St. John, *B. pseudopinnatum* W.H. Wagner, *B. pedunculosum* W.H. Wagner, *B. hesperium* (Maxon and R.T. Clausen) W.H. Wagner and Lellinger, *B. echo* W.H. Wagner, and *B. acuminatum* W.H. Wagner were all considered minor variants of *B. matricariifolium* A. Braun (Clausen, 1938), but are now recognized as species (Wagner and Wagner, 1981, 1983b, 1986, 1990b).

Plant groups for which morphological distinctions among species are subtle are appropriate candidates for molecular analyses. Although plastid inheritance is untested in Ophioglossaceae, molecular characters derived from plastid DNA often have the advantage of uniparental inheritance, either paternally (gymnosperms: Neale et al., 1989; Neale and Sederoff, 1988, 1989) or maternally (angiosperms: Harris and Ingram, 1991; ferns: Gastony and Yatskievych, 1992). Sequencing of the plastid gene *rbcL* has provided insight into higher order relationships among angiosperms (Chase et al., 1993), red algae (Freshwater et al., 1994), and ferns (Hasebe et al., 1995). Because of the conservative nature of *rbcL,* most studies have focused on relationships at the rank of genus or higher. Although *rbcL* has proved useful to address species-level questions in a limited number of plant groups (Price and Palmer, 1993; Williams et al., 1994), the extent to which *rbcL* can address species-level relationships remains largely unexplored. Furthermore, studies utilizing *rbcL* for phylogenetic reconstruction typically rely on single collections to represent entire species, even when these species have broad geographic distributions, apparently assuming that within-species polymorphism in *rbcL* is non-existent or that any polymorphism present will not influence phylogenetic inferences.

Representative species of the subgenera *Osmundopteris, Sceptridium,* and *Botrychium* from genus *Botrychium* had a mean *rbcL* sequence divergence value of 2.4% (Hauk, Parks, and Chase, unpubl. data), a value comparable to that observed among some angiosperm genera (Les, 1994). Sequence divergence

between two species of subgenus *Botrychium, B. lanceolatum* (J. Gmel.) Angstr. and *B. simplex* E. Hitchc., was 1.1%. These levels of divergence indicated that *rbcL* might be informative enough to address questions of phylogenetic relationships among species or species groups within subgenus *Botrychium*. Accordingly, three goals for the study were identified: 1) to investigate phylogenetic relationships among diploid *Botrychium* species and species groups, 2) to evaluate relationships among diploid and polyploid taxa in an effort to identify diploid progenitors of polyploid species, and 3) to assess within-species consistency of *rbcL* sequences across broad geographic distances.

Materials and Methods

DNA Extraction, Gene Amplification and Sequencing.—Plant material from 20 of the 24 currently recognized species of subgenus *Botrychium* was collected and silica-gel dried. Material of four species, *B. mormo* W.H. Wagner, *B. pallidum* W.H. Wagner, *B. boreale* Milde, and *B. gallicomontanum,* was not available for analysis. Table 2 contains collection sites and voucher information numbers. Total genomic DNA was extracted from approximately 0.03 to 0.50 grams of silica gel dried tissue that was ground at 65°C in 2× CTAB buffer (2% hexadecyl trimethyl ammonium bromide in 100 mM Tris-HCl pH 8.0 with 1.4 M NaCl and 20 mM EDTA) after Doyle and Doyle (1987), as modified by Wendel (1989) (20% polyvinylpyrrolidone, 5.0 mM ascorbic acid, 4.0 mM diethyldithiocarbamic acid sodium salt). Treatment with chloroform : isoamyl alcohol (24:1) removed nonpolar substances. Total DNA was purified by density gradient centrifugation in $CsCl_2$–ethidium bromide. After dialysis, the DNA was precipitated (1.0 M NaOAc, 70% EtOH) according to Sambrook et al. (1989) and spooled out of solution with a glass hook, rinsed in 70% ethanol, air dried, and resuspended in TE buffer pH 8.0. Amplification of the *rbcL* gene by standard methods (Saiki et al., 1987) used a forward primer (5′-ATGTCACCACAAACAGAAACTAAAGCAAGT-3′) that attached to the first 30 base pairs of the exon, and a reverse primer (5′-CTTCACAAGCAGCAGCTAGTTCAGGACTCC-3′) that began at the exon 3′ position 1352. Amplified products were purified using glass beads (Vogelstein and Gillespie, 1979). The purified products were sequenced directly using the dideoxynucleotide method of Sanger (1981). Both strands were sequenced using a combination of eight internal sequencing primers designed specifically for Ophioglossaceae (primer sequences available on request), plus the two amplification primers. A total of 1321 base pairs was obtained for each taxon (1351 minus the 30 base pairs of the forward primer). All substitutions unique to subgenus *Botrychium* species were recorded and verified by re-examination of the sequencing autoradiographs from both forward and reverse primers.

Data Analysis.—A total of 26 complete sequences, representing 20 species of subgenus *Botrychium,* was analyzed. Pairwise percent sequence divergences were calculated by dividing the number of nucleotide differences between two taxa by the total number of nucleotides sequenced (1321 for all sequences).

TABLE 2. Species of subgenus *Botrychium* used in *rbcL* analysis, collector and voucher citation, Genbank accession number and collection site. All Hauk vouchers are deposited at NCU. Wagner vouchers are deposited at MICH.

Species	Source/ voucher	Genbank number	Collection site
Botrychium lunaria	Moran 5426 (MO)	L40966	Taiwan
Botrychium lunaria	Hauk 564	L40965	Marathon, Ontario
Botrychium minganense	Hauk 578	L40970	Grand Sable Dunes, MI
Botrychium minganense	Hauk 566	L40971	Pic River, Ontario
Botrychium minganense	Hauk 598	L40968	Boulder Co., CO
Botrychium minganense	Hauk 584	L40969	Independence Pass, CO
Botrychium crenulatum	Hauk 616	L40959	Hurricane Creek, OR
Botrychium ascendens	Hauk 529	L40982	Hurricane Creek, OR
Botrychium spathulatum	Wagner 88036	L40980	Angler Settlement, Ontario
Botrychium spathulatum	Hauk 562	L40979	Marathon, Ontario
Botrychium simplex	Hauk 619	L40978	Mt. Ashland, OR
Botrychium simplex	Hauk 561	L40977	Grand Sable Dunes, MI
Botrychium pumicola	Hauk 618	L40976	Newberry Caldera, OR
Botrychium montanum	Hauk 607	L40916	Lake Co., MT
Botrychium lanceolatum var. *angustisegmentum*	Hauk 571	L40963	Taquamenon Falls, MI
Botrychium matricariifolium	Hauk 551	L40967	Grand Sable Dunes, MI
Botrychium hesperium	Hauk 552	L40960	Grand Sable Dunes, MI
Botrychium echo	Hauk 595	L40962	Echo Lake, CO
Botrychium acuminatum	Hauk 553	L40922	Grand Sable Dunes, MI
Botrychium pinnatum	Hauk 604	L40974	Stagger Inn, WA
Botrychium pseudopinnatum	Wagner 88037	L40975	Angler Settlement, Ontario
Botrychium pedunculosum	Hauk 615	L40973	Lostine River, OR
Botrychium paradoxum	Hauk 610	L40972	Waterton, Alberta
Botrychium ×*watertonense*	Hauk 611	L40981	Waterton, Alberta
Botrychium lineare	Hauk 581	L40964	Pike's Peak, CO
Botrychium campestre	Farrar s.n. (ISU)	L40961	Beamis Creek, IA

For cladistic analyses, *B. multifidum* (J. Gmel.) Rupr. and *B. lunaricides* (Michx.) Sw. of subgenus *Sceptridium* were chosen as outgroups, based on a larger *rbcL* analysis of Ophioglossaceae (Hauk, Parks, and Chase, unpubl. data). All sequences were analyzed using PAUP (Phylogenetic Analysis Using Parsimony) version 3.1.1 (Swofford and Begle, 1993). Fitch (1971; equal weights) parsimony analysis of 1000 random taxon entries was performed using Tree Bisection-Reconnection (TBR) swapping with MULPARS selected. One analysis was performed on *rbcL* sequences of diploid species and a second analysis was conducted with sequences of both diploid and polyploid species. The chromosome number for *B. lineare* W.H. Wagner is not known, but for purposes of analysis it was assumed to be diploid, based on isozyme banding patterns (Hauk, 1990). Bremer (1988) support (decay of parsimony) identified the tree lengths at which each branch collapsed into a polytomy. Figure 1 reports Bremer support values as a "d" preceeding a number (i.e., "d4" means that the branch decayed at four steps shorter than the length of the single most parsimonious tree). As a comparison to heuristic methods of analysis, I con-

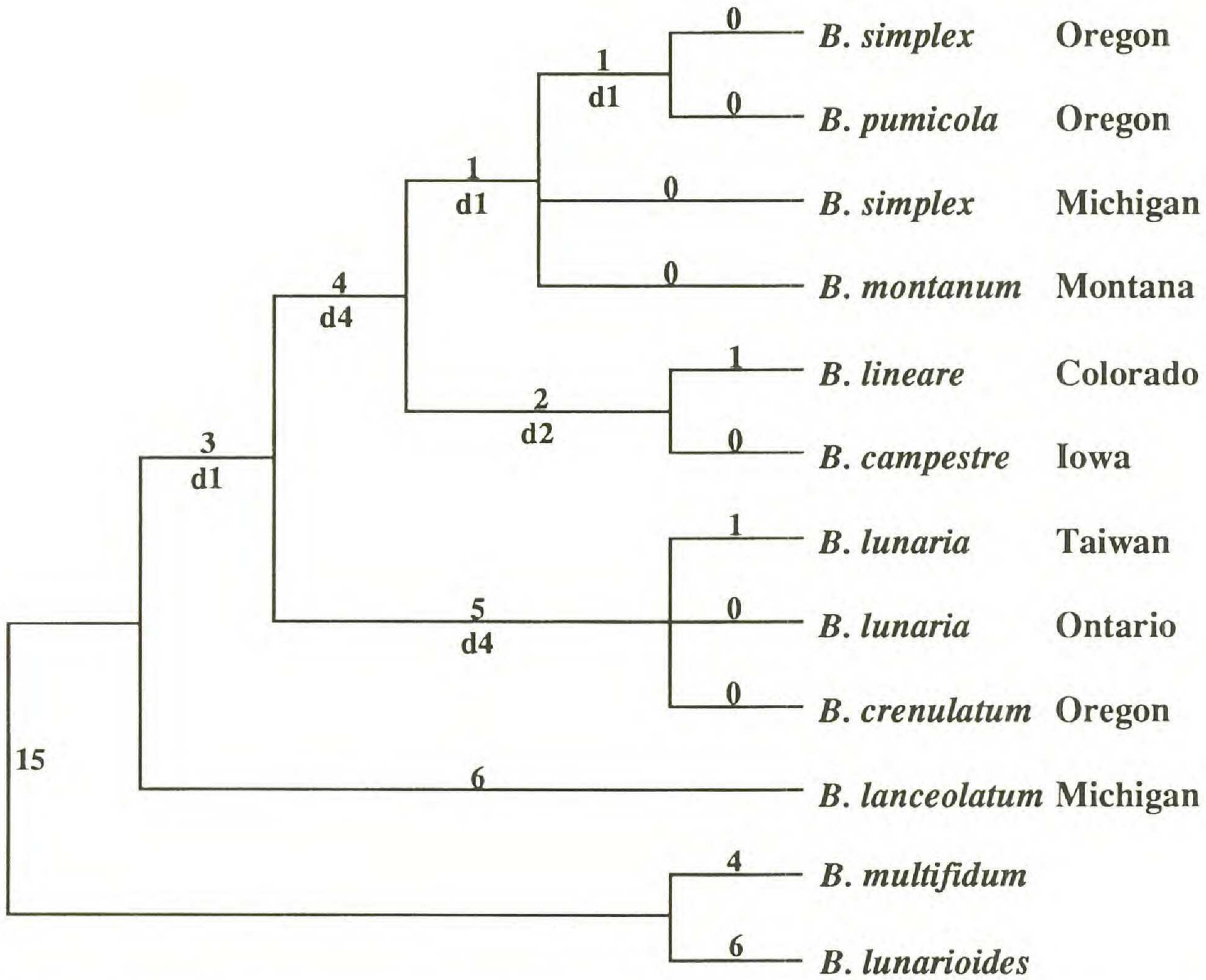

FIG. 1. The single most parsimonious Fitch tree for the analysis of ten *rbcL* sequences from eight diploid species of *Botrychium* subgenus *Botrychium*. All characters were equally weighted and unordered. Sequences of *B. multifidum* and *B. lunarioides* (subgenus *Sceptridium*) were used as outgroups. Segment lengths are listed above the branches, and the number of steps of relaxed parsimony required to collapse a branch are listed below the branches. The tree has a length of 49 steps, CI=0.918, RI=0.930, and HI=0.082.

ducted an exhaustive search of the 9 different *rbcL* sequences represented among the 20 species (i.e., some species had identical sequences).

RESULTS AND DISCUSSION

LEVELS OF SEQUENCE VARIATION IN *RBCL*.—Analysis of all sequences identified six species groups with identical *rbcL* sequences: 1) *B. lanceolatum* (var. *angustisegmentum* (Pease & A.H. Moore) R.T. Clausen), *B. acuminatum, B. echo, B. hesperium, B. matricariifolium, B. pedunculosum, B. pinnatum,* and *B. pseudopinnatum*; 2) *B. lunaria* (Ontario) and *B. crenulatum* W.H. Wagner; 3) *B. paradoxum* W.H. Wagner, *B.* ×*watertonense* W.H. Wagner, and all four populations of *B. minganense* Vict.; 4) *B. simplex* (MI) and *B. montanum* W.H. Wagner; 5) *B. simplex* (OR) and *B. pumicola* Cov.; and 6) *B. campestre* W.H. Wagner and Farrar and *B. spathulatum* W.H. Wagner. *Botrychium lunaria* (Taiwan) and *B. lineare* W.H. Wagner had the only two *rbcL* sequences that pos-

sessed autapomorphic mutations. The mean of pairwise comparisons of sequence divergence among the 9 different *rbcL* sequences distributed among 20 subgenus *Botrychium* species was 0.6%, a value much lower than the 1.87% reported for sister species of *Polypodium* (Haufler and Ranker, 1995). However, diploids representing each of the Wagner and Wagner sections had a mean sequence divergence of 1.1%, a level comparable to that observed among genera of aquatic cresses (Les, 1994). Levels of sequence divergence at a given rank will undoubtedly differ greatly among different groups of plants, due to different rates of mutation or contrasting concepts of taxonomy. Although the 0.6% mean sequence divergence among subgenus *Botrychium* species represents a very low level of divergence, statistics such as percentage sequence divergence do not in themselves provide a reliable evaluation of the information content of the divergence observed, or an indication of the strength of analyses based on them. Ritland and Eckenwalder (1992) estimated that for phylogenetic analyses optimal levels of sequence divergence occur around 10%, a point at which the number of single-hit substitution sites is maximized relative to the number of multiple-hit substitution sites. High levels of sequence divergence may lead to relationships that have poor internal and external support (Manhart, 1994). At lower levels of sequence divergence (<10%), the principle constraint for phylogenetic studies is identifying enough synapomorphies to resolve relationships (Les, 1994).

The absence of sequence divergence among many species of subgenus *Botrychium* indicates that *rbcL* is too conservative to address all species-level distinctions. However, the sequence variation detected among species groups should be considered informative, because it is associated with low levels of homoplasy (see below). The conservative nature of *rbcL* coupled with its putative uniparental pattern of inheritance should reveal clear patterns of relationships, first, among diploid species groups and, second, between polyploids and their diploid chloroplast progenitor.

PHYLOGENETIC ANALYSES.—Forty-three variable nucleotide sites were detected among the 22 species included in this study. Of these 43 variable sites, 24 were variable within subgenus *Botrychium*. Sixteen sites were informative in the analysis of diploid species and 22 were informative in the combined analysis of diploids and polyploids. For diploid species, Fitch parsimony analysis produced a single most parsimonious tree with a length of 49 steps (Fig. 1), a consistency index (CI) of 0.918, a retention index (RI) of 0.930 and a homoplasy index (HI) of 0.082 when both informative and uninformative characters were used. When only phylogenetically informative characters were included, a tree of 34 steps resulted with a CI of 0.882, a RI of 0.930 and a HI of 0.118. In the second analysis, which included sequences of both diploid and polyploid species, a single most parsimonious tree of 49 steps was produced (Fig. 2) with a CI of 0.918, a RI of 0.974 and a HI of 0.082. When uninformative characters were excluded the tree had 41 steps, a CI of 0.902, a RI of 0.974 and a HI of 0.098. The exhaustive search yielded the same topology as the heuristic analysis of all taxa.

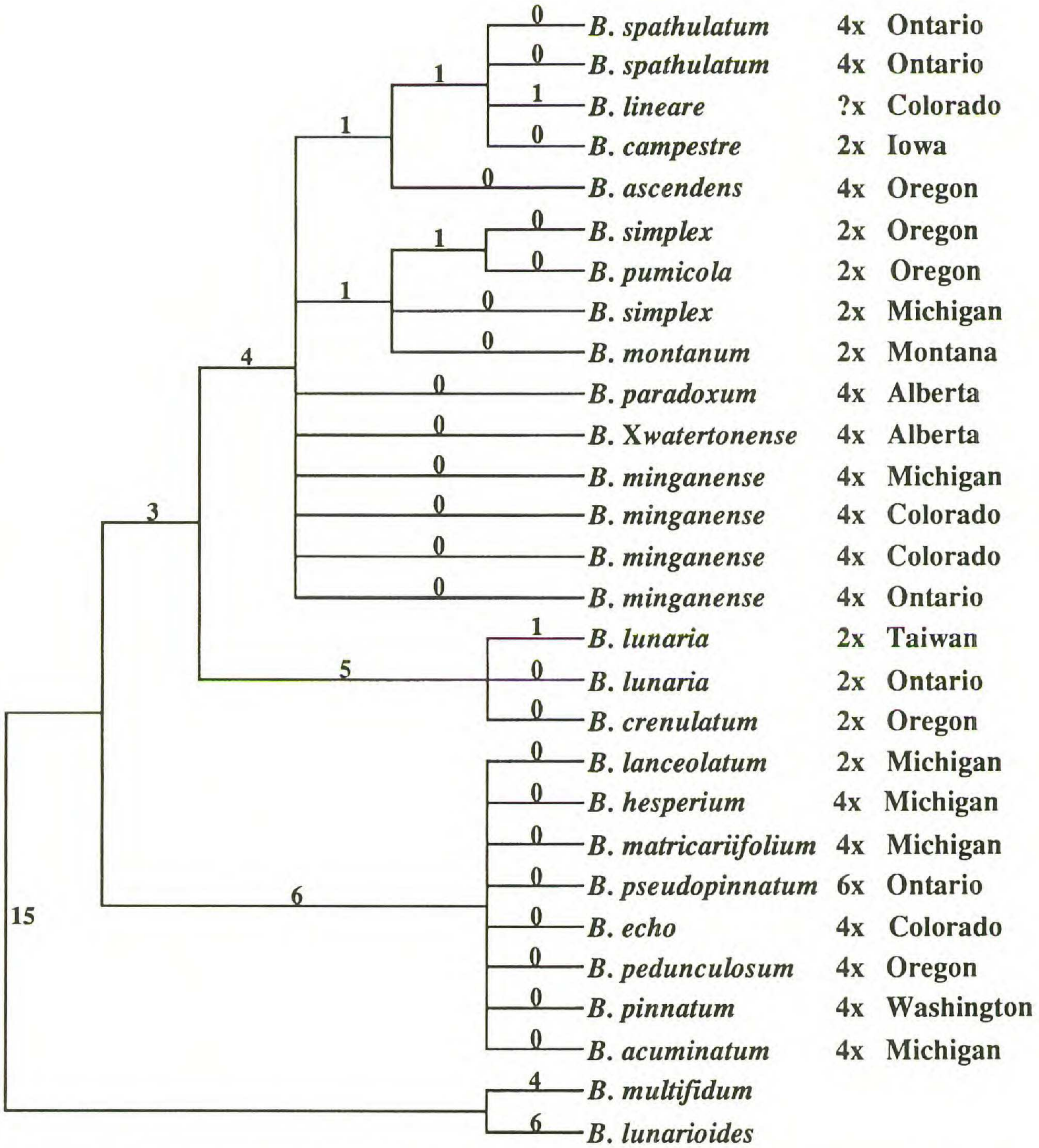

FIG. 2. The single most parsimonious Fitch tree from the analysis of 26 *rbcL* sequences from 20 species of *Botrychium* subgenus *Botrychium*. All characters were equally weighted and unordered. Sequences of *B. multifidum* and *B. lunarioides* (subgenus *Sceptridium*) were used as outgroups. Segment lengths are listed above the branches, and the number of steps of relaxed parsimony required to collapse a branch are listed below the branches. The tree has a length of 49 steps, CI=0.918, RI=0.974, and HI=0.082.

Within subgenus *Botrychium* there are three well-supported diploid species clades (Fig. 1): The lanceolatum clade (one species), the lunaria clade (two species), and the simplex-campestre clade (five species). The lunaria and simplex-campestre clades collapse on a consensus tree four or more steps longer than the shortest tree (d4). Within the simplex-campestre clade there were two

less well-supported subclades: The simplex subclade (three species, d1) and the campestre subclade (two species, d2). Within the simplex subclade, two populations of *B. simplex* differed by a single substitution. *Botrychium simplex* from Oregon and *B. pumicola* had identical sequences, as did *B. simplex* from Michigan and *B. montanum* from Montana. Two species, *B. lunaria* and *B. crenulatum,* formed the lunaria clade.

The second analysis included sequences of both diploid and polyploid subgenus *Botrychium* species (Fig. 2). Most *rbcL* sequences of polyploid species were identical to that of one of the diploid species. The polyploid species, *B. pinnatum, B. pseudopinnatum, B. matricariifolium, B. hesperium, B. echo, B. pedunculosum,* and *B. acuminatum,* had *rbcL* sequences identical to the diploid *B. lanceolatum,* and both populations of the tetraploid species, *B. spathulatum,* shared the same *rbcL* sequence with *B. campestre.* Four polyploid subgenus *Botrychium* species did not share identical *rbcL* sequences with any diploid species sampled. The tetraploid species, *B. ascendens* W.H. Wagner, associated with the campestre subclade, but lacked one of the two synapomorphies that defined the subclade. Three other taxa, *B. minganense, B. paradoxum,* and *B. ×watertonense* did not possess synapomorphies of the campestre or simplex subclades, but nevertheless possessed the four synapomorphies that defined the simplex-campestre clade. The analysis detected no autapomorphic substitutions that distinguished *B. paradoxum* from *B. ×watertonense* or among the four populations of *B. minganense.*

THE MONOPHYLY OF SUBGENUS *BOTRYCHIUM.*—Species of subgenus *Botrychium* possess a suite of morphological and anatomical characters that distinguish them collectively from other subgenera of genus *Botrychium* (Bower, 1926; Clausen, 1938; Tryon and Tryon, 1982). The small size, pale color, fleshy leaves, pinnately compound trophophores (except *B. lanceolatum,* see below), invariable presence of a fertile segment, and ephemeral leaf growth support the monophyly of the subgenus. Micromorphological characters, such as similar pollen architecture (Tryon and Lugardon, 1991) and a leaf sheath covering with a fused slit (Kato, 1987), have been reported as synapomorphies for the group. Reports of hybrids among many species document the close genetic relationship among species (Wagner, 1980, 1991; Wagner et al., 1985; Wagner and Wagner, 1988). No reports of hybrids between members of subgenus *Botrychium* and the often sympatric subgenera *Sceptridium* and *Osmundopteris* are known, and further suggest isolating mechanisms among the subgenera.

In an *rbcL* analysis of Ophioglossaceae, all species of subgenus *Botrychium* form a monophyletic group (Hauk, Parks and Chase, unpubl. data). The addition of various outgroups within and outside of Ophioglossaceae does not change the strong support for the monophyly of subgenus *Botrychium.* Although no morphological data are included in the analysis, all evidence, morphological and molecular, is consistent with the hypothesis that subgenus *Botrychium* is monophyletic.

RELATIONSHIPS AMONG DIPLOID SPECIES OF SUBGENUS *BOTRYCHIUM*.—Of the three major clades in the *rbcL* analysis, the lanceolatum clade is sister to the rest of the subgenus. The lunaria clade (two diploid species) is sister to the simplex-campestre clade (the remaining five diploid species). Relationships among these three major clades are consistent with ideas of relationship based on morphology. Diploid *B. lanceolatum* is the only species in subgenus *Botrychium* that exhibits ternate division of the sterile segment, as do species of subgenera *Sceptridium, Japanobotrychium* and *Osmundopteris*. If the presence of ternate leaf organization in *B. lanceolatum* is ancestral and the pinnate leaf organization of the remaining species is derived, then morphological patterns are consistent with the *rbcL* data.

The lunaria clade contains the diploid species *B. lunaria* and *B. crenulatum*, which are morphologically similar due to oppositely arranged, broadly fan-shaped pinnae. The lunaria clade is sister to the simplex-campestre clade. Within the latter, subclade simplex contains the diploids *B. simplex, B. pumicola* and *B. montanum*, all of which share a cup-like curve in the terminal pinna of the sterile segment. The campestre subclade contains the diploid species *B. campestre* and the recently described *B. lineare*, for which ploidy is not known. A close relationship between *B. campestre* and *B. lineare* is evident from morphology (Wagner, 1994), and the two species differ by only a single autapomorphic substitution in *B. lineare*. Thus, each of the diploid clades identified by *rbcL* analysis contains species that share similar morphological features. However, fundamental phylogenetic relationships among these diploid clades are difficult to establish based on morphological characters, because few characters exist and, of those, few are informative regarding relationships among clades.

Studies of isozyme variation among closely related species are useful to establish basic ideas of relationship (Crawford, 1983; Werth, 1989), although these data usually have been analyzed phenetically, and therefore do not specifically address cladistic relationships. Nonetheless, hypotheses of relationships based on isozyme genetic identities (Nei, 1972) of six diploid species of subgenus *Botrychium* (Hauk, 1990) corroborate ideas of relationships based on parsimony analysis of *rbcL* sequences (Fig. 3). Diploid species with similar *rbcL* sequences tend to have higher isozymic genetic identities than diploids with divergent *rbcL* sequences. For example, diploid species *B. crenulatum* shares a genetic identity of 0.53 with *B. lunaria*, the only other member of its *rbcL* clade. Species from other *rbcL* clades, such as *B. simplex* (0.34) and *B. lanceolatum* (0.39), have lower genetic identities with *B. crenulatum* than does *B. lunaria*. *Botrychium lineare*, a member of the simplex-campestre clade, has higher identities with *B. simplex* (0.59) and *B. pumicola* (0.83) than it does to species in other *rbcL* clades such as *B. lunaria* (0.24) and *B. lanceolatum* (0.04). The degree of correlation between isozyme and *rbcL* data supports the assertion that *rbcL* is informative concerning phylogenetic relationships of species groups in subgenus *Botrychium*.

INTRASPECIFIC VARIATION IN *RBCL* SEQUENCES OF DIPLOID SPECIES.—Because most

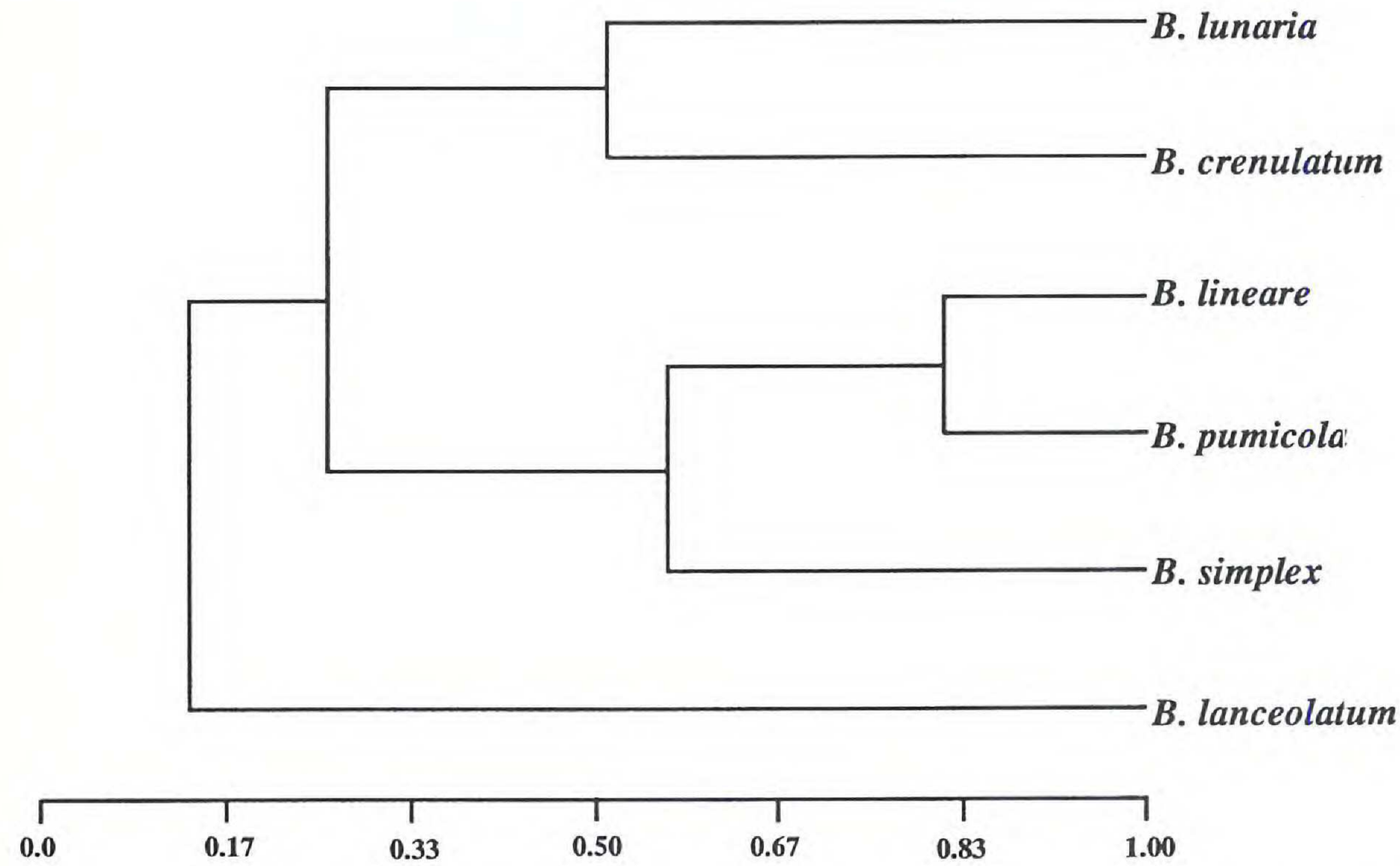

FIG. 3. Cluster analysis of six diploid species of *Botrychium* subgenus *Botrychium*, based on Nei's (1972) genetic identity of isozyme data, using the unweighted pair-group method (UPGMA). The scale represents the range of potential genetic identity values.

studies utilizing *rbcL* sequences focus on higher order relationships among genera and families, sometimes one accession of a single individual is assumed to represent a species (or even a genus or a family). At low levels of relationship (i.e., among species), where sequence divergence is minimal, within-species variation in *rbcL* may confuse phylogenetic relationships. One consequence of within-species *rbcL* sequence variation may be fixation of different base pair substitutions in different populations across the range of the species. Although few studies have investigated the possibility of within-species variation in *rbcL,* species with broad distributions have the potential to possess more than one *rbcL* sequence.

Two geographically widespread diploid species from subgenus *Botrychium* were selected for intraspecific sampling: *B. simplex* and *B. lunaria.* Intraspecific morphological variation is evident in *B. simplex* in which individuals collected at a single site may show considerable variation in the size and complexity of the lowermost pinnae pair. Some *B. simplex* individuals have large, well developed basal pinnae that are as dissected as the main axis of the sterile segment, whereas other individuals show no lobing or enlargement of lower pinnae. Historically, eastern and western varieties of *B. simplex* have been recognized (Clausen, 1938; Wagner and Wagner, 1993). For this study one population from Michigan represents the eastern form and a population from Oregon represents the western form. *Botrychium lunaria* is the most geographically widespread species in subgenus *Botrychium*; collections are reported from nearly every continent (Clausen, 1938). The potential for long distance

dispersal is high in this species. Two widely disjunct populations, one from Michigan and one from Taiwan, represent the range of *B. lunaria.*

Botrychium lunaria from Ontario does not possess the autapomorphic substitution that distinguishes the Taiwan population of *B. lunaria* from *B. crenulatum.* If within-species sampling had not been conducted, then *B. lunaria* and *B. crenulatum* would appear differentiated by a single substitution. Thus, the North American collections of *B. lunaria* and *B. crenulatum* are more similar to each other than the two populations of *B. lunaria* collected from widely separate geographic areas, Ontario and Taiwan. Several explanations for this pattern of relationship are possible: 1) *B. crenulatum* and *B. lunaria* may share a common ancestor that gave rise to geographically proximal, but morphologically distinct species with identical *rbcL* sequences, whereas geographically disjunct populations of *B. lunaria* are morphologically indistinguishable but have divergent *rbcL* sequences; 2) introgression of the lunaria chloroplast type into *B. crenulatum* may explain the lack of *rbcL* sequence divergence between *B. lunaria* and *B. crenulatum*; 3) lineage sorting of chloroplast DNA polymorphisms could produce the observed pattern of relationship between *B. lunaria* and *B. crenulatum* (Doyle et al., 1990). According to the lineage sorting hypothesis, *rbcL* polymorphisms were present in the common ancestor of *B. lunaria* and *B. crenulatum,* and both species inherited the polymorphisms. The Ontario population of *B. lunaria* became fixed for one *rbcL* pattern and the Tiawan population for a second *rbcL* pattern. By chance, *B. crenulatum* became fixed for the same *rbcL* pattern as the Ontario population of *B. lunaria,* thus giving the appearance of most recent shared ancestry between *B. crenulatum* and the Michigan population of *B. lunaria.*

Relationships among *B. pumicola, B. montanum,* and the two populations of *B. simplex* are more complex. The western population of *B. simplex* shares a substitution with the western species, *B. pumicola,* whereas the eastern population of *B. simplex* lacks this single substitution, as does *B. montanum,* another western North American species. The *rbcL* tree indicates that the common ancestor of the simplex subclade gave rise to *B. montanum* and *B. simplex* directly. Eastern and western populations of *B. simplex* then diverged, and the western population produced *B. pumicola.* Alternatively, phylogenetic sorting (Doyle et al., 1990) or introgression (Rieseberg, et al., 1990; Rieseberg and Brunsfeld, 1991) may account for the same patterns of relationship, although without further information from nuclear markers this hypothesis cannot be evaluated.

The intraspecific variation detected in *B. lunaria* and *B. simplex* significantly influences the interpretation of the relationship of these two species to that of closely related species. In both *B. simplex* and *B. lunaria,* sampling of a single population would have resulted in erroneous interpretations of species relationships. Furthermore, these data suggest the possibility of undetected *rbcL* variation in these and other diploid subgenus *Botrychium* species.

Associations of Diploid and Polyploid Species.—The ancestry of many putative allopolyploid species of subgenus *Botrychium* is enigmatic, because

TABLE 3. Hypothetical parents for polyploid species of subgenus *Botrychium* (after F.S. Wagner, 1993).

Polyploid species	Hypothetical parents
B. minganense	*B. lunaria* × *B. pallidum*
B. spathulatum	*B. lunaria* × *B. campestre*
B. ascendens	*B. crenulatum* × *B. montanum*
B. gallicomontanum	*B. simplex* × *B. campestre*
B. pinnatum	*B. lanceolatum* × *B. lunaria*
B. hesperium	*B. lanceolatum* × *B. simplex*
B. echo	*B. lanceolatum* × *B. campestre*
B. pedunculosum	*B. lanceolatum* × *B. montanum*
B. matricariifolium	*B. lanceolatum* × *B. pallidum*
B. acuminatum	metaspecies of *B. matricariifolium*
B. pseudopinnatum	*B. pinnatum* × *B. simplex*
B. ×*watertonense*	*B. paradoxum* × *B. hesperium*

there are relatively few discrete morphological characters that distinguish taxa. Hybridization followed by polyploidy blurs species specific characters and makes the recognition and identification of species difficult. Assuming uniparental inheritance of the chloroplast genome, recent polyploid derivatives of extant diploid species should possess similar or identical *rbcL* sequences to known diploids. Polyploid species of subgenus *Botrychium* associate with various diploid species in the *rbcL* tree (Fig. 2) and these associations are largely congruent with previously hypothesized ideas of polyploid origin (Wagner, 1993; Table 3).

The lanceolatum clade contains seven polyploid species and a single diploid, *B. lanceolatum*. The seven polyploid species have *rbcL* sequences identical to that of *B. lanceolatum* and, based on morphological studies, are putative allopolyploid derivatives of *B. lanceolatum* and pinnate diploids (Wagner, 1993). Thus, *B. lanceolatum* appears to be the chloroplast parent of the seven polyploid species in the lanceolatum clade. Sequence divergence in *rbcL* almost certainly does not coincide with all speciation events, and is thus not useful to distinguish all subgenus *Botrychium* species. A relatively recent origin for each of the polyploid species in the lanceolatum clade could explain why none of the seven polyploids have autapomorphic substitutions and yet all possess the six substitutions that distinguish *B. lanceolatum* from other diploid species.

The second major *rbcL* clade is the lunaria clade, which includes only the diploids *B. lunaria* and *B. crenulatum*. *Botrychium crenulatum* was the only diploid in addition to *B. lunaria* placed in section *Lunaria* by Wagner and Wagner (pers. comm.). Although morphological evidence exists for *B. lunaria* as one diploid progenitor of *B. spathulatum*, *B. minganense*, *B. pinnatum*, and *B. pseudopinnatum* (via *B. pinnatum*), none of the polyploids possess the *rbcL* sequence of *B. lunaria*. Because *B. lunaria* is the most geographically widespread of all subgenus *Botrychium* species, it is surprising that no polyploids share its *rbcL* sequence, but further sampling among putative polyploid deriv-

atives of *B. lunaria* might uncover examples of inheritance of the lunaria *rbcL* sequence.

The simplex-campestre clade contains ten species, five of which are polyploids. The clade is composed of three distinct groups, two poorly supported subclades (d1) and three taxa that did not associate with either subclade. The campestre subclade contains the diploid *B. campestre,* the tetraploids *B. spathulatum* and *B. ascendens,* and one additional species whose ploidy level is not known, *B. lineare.* All four species exhibit the common feature of narrow, almost linear pinnae, with the possible exception of *B. spathulatum,* in which the broader pinnae shape is probably due to the influence of *B. lunaria* as one of its diploid parents (Wagner, 1993). The identical *rbcL* sequences of *B. spathulatum* and *B. campestre* implicate *B. campestre* as the chloroplast parent of *B. spathulatum.* In this case the more geographically restricted and less common diploid parent, *B. campestre,* contributed its chloroplast genome to the polyploid.

The association of *B. ascendens* with the campestre subclade was not suggested in previous morphological investigations (Wagner, 1993; Table 3), although all members of the subclade possess narrow pinnae. *Botrychium ascendens* does not possess both of the synapomorphies of the campestre subclade. The lack of one synapomorphy may be due to early divergence of *B. ascendens* from the common ancestor of *B. campestre, B. lineare,* and *B. spathulatum.* Alternatively, an unsampled diploid with the *B. ascendens* chloroplast type may exist.

The tetraploids, *B. minganense, B. paradoxum* and *B. ×watertonense* do not possess the synapomorphies that distinguish the simplex and campestre subclades and appear to retain the ancestral *rbcL* sequence of the entire simplex-campestre clade. There are at least three explanations for the lack of association of these tetraploid species with any known diploid: 1) *B. minganense, B. paradoxum* and *B. ×watertonense* have reversions of synapomorphies that establish the campestre and simplex subclades as distinct (an explanation that seems unlikely given the conservative nature of *rbcL*); 2) *B. minganense, B. paradoxum,* and *B. ×watertonense* may have diverged from the common ancestor of the simplex-campestre clade, and thus do not possess the synapomorphies of either the simplex or campestre subclades; 3) *B. minganense, B. paradoxum* and *B. ×watertonense* may possess the chloroplast genome of a diploid that was not sampled in this analysis (*B. pallidum*) or is extinct. Material of the diploid *B. pallidum,* a potential member of this clade, should be available in the near future. Only extensive collections and continued fieldwork by experienced collectors can address the possibility of the existence of an unknown diploid.

The patterns of relationship between diploids and polyploids reveal several interesting speciation trends in subgenus *Botrychium.* First, certain diploids (*B. lanceolatum* and *B. campestre*) are frequently involved as the chloroplast parent in hybridization events leading to polyploidy. Second, species such as *B. lunaria, B. crenulatum,* and three species of the simplex subclade (*B. simplex, B. montanum, B. pumicola*) do not appear to be chloroplast parents of

any polyploids sampled. Members of the lunaria clade and the simplex subclade appear to be speciating at the diploid level while presumably contributing only nuclear genomes to the polyploids. Third, the campestre subclade is the only group containing both closely related diploid species and a polyploid derivative possessing the *rbcL* pattern of a diploid subclade member. Reasons for these patterns of relationship are enigmatic. Differential production of antheridia and archegonia in gametophytes of different diploid species could account for these patterns, although no data concerning overall production of antheridia and archegonia in gametophytes of subgenus *Botrychium* are available at this time.

INTRASPECIFIC VARIATION IN POLYPLOID SPECIES.—Within-species plastid DNA variation in allopolyploid taxa may originate from reciprocal crosses when there are multiple hybridization events between progenitor diploids (Gibby, 1977; Werth et al., 1985; Haufler and Soltis, 1986; Barrington et al., 1989; Soltis and Soltis, 1989; Stein and Barrington, 1990). Different parents may contribute their chloroplast genome to the resulting hybrids. Thus, conspecific populations of allopolyploids may possess both parental chloroplast genomes (Stein and Barrington, 1990). In the tetraploid, *B. minganense,* a high level of morphological variation may indicate the possibility of unrecognized cryptic species (W. H. Wagner, pers. comm.). High amounts of isozymic variation among eleven *B. minganense* populations lends support to the hypothesis of a complex origin for *B. minganense* (Hauk, 1990). In order to test for different chloroplast genomes in *B. minganense,* four populations, two from the Great Lakes region and two from Colorado, were sampled (Table 2). In contrast to the reports of high levels of intraspecific morphological and isozymic variation, all four populations of *B. minganense* share identical *rbcL* sequences. This lack of *rbcL* variation is consistent with the hypothesis of a single plastid origin for eastern and western *B. minganense* populations.

Bimodal morphological variation is apparent in the putative allotetraploid, *B. spathulatum*; different individuals may strongly resemble one or the other of the putative parents, *B. campestre* or *B. lunaria* (Hauk, unpubl. data), perhaps as a result of introgression. Two populations of *B. spathulatum* were collected from southern Ontario, one which more closely resembled *B. campestre* and the other which strongly resembled *B. lunaria.* Sequences from both *B. spathulatum* populations are identical to the *rbcL* sequence of *B. campestre,* and are consistent with a single origin for these populations of *B. spathulatum.* The lack of *rbcL* variation in *B. minganense* and *B. spathulatum* indicates that hybridization events leading to allopolyploid speciation are extremely rare and/or consistently favor one parent as the chloroplast donor.

COMPARISONS OF *RBCL* TREE AND MORPHOLOGICAL IDEAS OF RELATIONSHIP.—The Wagner and Wagner (pers. comm.) classification of subgenus *Botrychium* differs from the *rbcL* tree in four ways (Table 4): 1) the *rbcL* tree fuses the Wagner and Wagner sections *Lanceolatum* and *Matricariifolium*; 2) the *rbcL* tree supports the establishment of a new section of subgenus *Botrychium,* provisionally referred to as "section *Campestre*" pending further morphological study

TABLE 4. Contrasting sectional classifications for species of *Botrychium* subgenus *Botrychium*, based on Wagner and Wagner's (pers. comm.) most recent, as yet not fully published system and the present *rbcL* analysis. Species not sampled for the *rbcL* analysis are indicated with a dash (—). Note that "section *Campestre*" is a provisional name.

Species	Wagner and Wagner (sectional placement)	*rbcL* analysis (sectional placement)
B. lanceolatum	*Lanceolatum*	*Lanceolatum*
B. matricariifolium	*Matricariifolium*	*Lanceolatum*
B. acuminatum	*Matricariifolium*	*Lanceolatum*
B. hesperium	*Matricariifolium*	*Lanceolatum*
B. echo	*Matricariifolium*	*Lanceolatum*
B. pedunculosum	*Matricariifolium*	*Lanceolatum*
B. pinnatum	*Matricariifolium*	*Lanceolatum*
B. pseudopinnatum	*Matricariifolium*	*Lanceolatum*
B. boreale	*Matricariifolium*	—
B. simplex	*Simplex*	*Simplex*
B. pumicola	*Simplex*	*Simplex*
B. montanum	*Simplex*	*Simplex*
B. mormo	*Simplex*	—
B. gallicomontanum	*Simplex*	—
B. lunaria	*Lunaria*	*Lunaria*
B. crenulatum	*Lunaria*	*Lunaria*
B. pallidum	*Lunaria*	—
B. ascendens	*Lunaria*	*"Campestre"*
B. spathulatum	*Lunaria*	*"Campestre"*
B. minganense	*Lunaria*	Status undetermined
B. paradoxum	Status undetermined	Status undetermined
B. ×watertonense	Status undetermined	Status undetermined
B. lineare	Status undetermined	*"Campestre"*
B. campestre	Status undetermined	*"Campestre"*

and characterization, which would include *B. campestre*, *B. lineare*, and *B. spathulatum*; 3) *B. ascendens* aligns with "section *Campestre*" and not with one of its hypothetical diploid parents, *B. crenulatum* or *B. montanum*; 4) *B. minganense* does not associate with members of section *Lunaria* in the *rbcL* tree, and its sectional affinity is unknown, although it groups closely with *B. paradoxum* and *B. ×watertonense*.

Despite these differences, the *rbcL* tree of subgenus *Botrychium* reflects to a degree the relationships as hypothesized by the morphological studies of Wagner and Wagner. The major discrepancies between the two classifications may be attributed to problems associated with interpretation of biparentally inherited morphological characters versus uniparentally inherited *rbcL* sequences. For example, the *rbcL* tree places *B. ascendens*, *B. spathulatum*, and *B. minganense* outside section *Lunaria*, although either *B. lunaria* or *B. crenulatum* is probably involved in the parentage of these polyploid species, but not as the chloroplast donor. Morphological phenotypes of allopolyploids would not be expected to more closely resemble the chloroplast parent than the non-chloroplast parent, i.e., the allopolyploids would be placed closest to the par-

ent to which the closest morphological resemblance occurs, whether or not it is the chloroplast donor.

Classification of hybrids and allopolyploid taxa is particularly problematic if the parental taxa belong to different taxonomic groups (Kellogg, 1989; Wagner, 1983), because an allopolyploid species does not belong exclusively to the taxonomic group of either parent. Debate concerning the treatment of hybrids and allopolyploids in classifications has produced at least three possible solutions to the dilemma (Wagner, 1983): a) placement of hybrid or allopolyploid species next to the first parent appearing in the classification and reference to the hybrid or allopolyploid again when the second parent is listed; b) placement of all hybrids and allopolyploids in a special section at the end of the species treatment; or c) arbitrary placement of hybrids and allopolyploids either with the first-listed parent or in a special section, based on characters such as fertility versus sterility, low versus high abundance, or other factors deemed justifiable by the researcher.

These *rbcL* data provide strong evidence of the probable chloroplast parent of many subgenus *Botrychium* polyploids, although identification of the non-chloroplast parent(s) remains uncertain for many species, due to the inherent limitations of interpreting cryptic morphological differences that are further obscured by hybridization events. Other subgenus *Botrychium* species (*B. minganense, B. paradoxum,* and *B. ascendens*) do not have demonstrable chloroplast progenitors, and their origin remains a mystery. The most appropriate solution to the problem of classifying subgenus *Botrychium* polyploids is the creation of a special section in which all putative allopolyploids are placed. The allopolyploid section should be retained until more rigorous evidence elucidates the non-chloroplast progenitor of these species. Only when a clear understanding of the ancestry of these species is available will it be possible to create a classification system that reflects the evolutionary history of allopolyploid species of subgenus *Botrychium.*

Conclusions

Even low amounts of sequence divergence among species (0.6% in subgenus *Botrychium*) can contain phylogenetically informative signal if coupled with low levels of homoplasy. The divergence detected among species of subgenus *Botrychium* demonstrates that *rbcL* is more applicable to species-level questions than previously thought, although certainly not all species-level distinctions may be resolved. In the absence of more variable yet easily studied genes, *rbcL* can provide basic evidence concerning phylogenetic relationships among species and species groups. Within subgenus *Botrychium,* analysis of *rbcL* data provides the first opportunity to establish fundamental phylogenetic relationships among a confusing assemblage of morphologically cryptic species. In addition to supporting the monophyly of subgenus *Botrychium,* the *rbcL* phylogeny identified *B. lanceolatum* as sister to all other diploid species, and the group composed of *B. lunaria* and *B. crenulatum* as sister to the five diploid species of the simplex-campestre clade (*B. campestre, B. lineare, B. simplex,*

B. montanum, and *B. pumicola*). The *rbcL* phylogeny supports the establishment of a new section (provisionally called "section *Campestre*") containing *B. campestre*, *B. lineare*, and *B. spathulatum*. Relationships based on *rbcL* analyses are largely consistent with morphological and isozymic hypotheses of relationship.

In groups possessing polyploids of uncertain origin, *rbcL* can provide strong evidence of the chloroplast parent of each polyploid, provided that the diploids are well differentiated. Analysis of *rbcL* sequences establishes affinities of polyploid species of subgenus *Botrychium* to their diploid chloroplast donors, and thus provides the strongest evidence to date concerning polyploid origins. The within-species *rbcL* variation detected in *B. lunaria* and *B. simplex* demonstrates that intraspecific *rbcL* variation can significantly influence interpretations of species relationships. Population sampling for studies of species relationships may be necessary to avoid misinterpretation of phylogenetic relationships.

ACKNOWLEDGMENTS

I would like to thank Herb Wagner, Florence Wagner, Robbin Moran, and Don Farrar for providing plant material. For critical reading of the manuscript and helpful suggestions, I would like to thank Mark Chase, Michael Fay, Cynthia Morton, Florence Wagner, Charlie Werth, and Paul Wolf. This work was supported by a National Science Foundation Doctoral Dissertation Improvement Grant (BSR-9224287).

LITERATURE CITED

BARRINGTON, D. S., C. H. HAUFLER, and C. R. WERTH. 1989. Hybridization, reticulation, and species concepts in the ferns. Amer. Fern J. 79:55–64.

BIERHORST, D. W. 1971. *Morphology of vascular plants.* Macmillan Company, New York.

BOWER, F. O. 1926. *The ferns (Filicales).* Vol. II, Cambridge University Press, Cambridge.

BREMER, K. 1988. The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. Evolution 42:795–803.

CLAUSEN, R. T. 1938. A monograph of the Ophioglossaceae. Mem. Torrey Bot. Club. 19:1–177.

CHASE, M. W., D. SOLTIS, R. G. OLMSTEAD, D. MORGAN, D. H. LES, B. MISHLER, M. R. DUVALL, R. A. PRICE, H. G. HILLS, Y-L. QIU, K. A. KRON, J. H. RETTIG, E. CONTI, J. D. PALMER, J. R. MANHART, K. J. SYTSMA, H. J. MICHAELS, W. J. KRESS, K. G. KAROL, W. D. CLARK, M. HEDRÉN, B. S. GAUT, R. K. JANSEN, K-J. KIM, C. F. WIMPEE, J. F. SMITH, G. R. FURNIER, S. H. STRAUS, Q-Y. XIANG, G. M. PLUNKETT, P. S. SOLTIS, S. M. SWENSON, S. E. WILLIAMS, P. A. GADEK, C. J. QUINN, L. EGUIARTE, E. GOLENBERG, G. H. LEARN, S. W. GRAHAM, S. C. H. BARRETT, S. DAYANANDAN, and V. A. ALBERT. 1993. Phylogenetics of seed plants: An analysis of nucleotide sequences from the plastid gene *rbc*L. Ann. Missouri Bot. Gard. 80:528–580.

CRAWFORD, D. J. 1983. Phylogenetic and systematic inferences from electrophoretic studies. Pp. 257–287 *in* S. D. Tanksley and T. J. Orton, eds. *Isozymes in plant genetics and breeding*, Part A. Elsevier Science Publishers, Amsterdam.

DOYLE, J. J., J. L. DOYLE, and A. H. D. BROWN. 1990. Chloroplast DNA polymorphism and phylogeny in the B genome of *Glycine* subgenus *Glycine*. Amer. J. Bot. 77:772–782.

FARRAR, D. R., and C. L. JOHNSON-GROH. 1991. A new prairie moonwort (*Botrychium* subgenus *Botrychium*) from northwestern Minnesota. Amer. Fern J. 81:1–6.

FITCH, W. M. 1971. Toward defining the course of evolution: Minimum change for a specific tree topology. Syst. Zool. 20:406–416.

FOSTER, A. S., and E. M. GIFFORD. 1974. *Comparative morphology of vascular plants.* W. H. Freeman and Company, San Francisco.

FRESHWATER, D. W., S. FREDERICQ, B. S. BUTLER, M. H. HOMMERSAND, and M. W. CHASE. 1994. A gene phylogeny of the red algae (Rhodophyta) based on plastid *rbcL.* Proc. Natl. Acad. Sci. U.S.A. 91:7281–7285.

GASTONY, G. J., and G. YATSKIEVYCH. 1992. Maternal inheritance of the chloroplast and mitochondrial genomes in cheilanthoid ferns. Amer. J. Bot. 79:716–722.

GIBBY, M. 1977. The origin of *Dryopteris campyloptera.* Canad. J. Bot. 55:1419–1428.

HARRIS, S. A., and R. INGRAM. 1991. Chloroplast DNA and biosystematics: The effects of intraspecific diversity and plastid transmission. Taxon 40:393–412.

HASEBE, M., P. G. WOLF, K. M. PRYER, K. UEDA, M. ITO, R. SANO, G. J. GASTONY, J. YOKOYAMA, J. R. MANHART, N. MURAKAMI, E. H. CRANE, C. H. HAUFLER, and W. D. HAUK. 1995. Fern phylogeny based on *rbcL* nucleotide sequences. Amer. Fern J. 85:134–181.

HAUFLER, C. H., and D. S. SOLTIS. 1986. Genetic evidence indicates that homosporous ferns with high chromosome numbers may be diploid. Proc. Natl. Acad. Sci. U.S.A. 83:4389–4393.

HAUFLER, C. H., and T. A. RANKER. 1995. *RbcL* sequences provide phylogenetic insights among sister species of the fern genus *Polypodium.* Amer. Fern J. 85:361–374.

HAUK, W. D. 1990. A molecular analysis of evolutionary patterns and reproductive processes in the genus *Botrychium.* Master's thesis. University of Kansas, Lawrence, KS.

KATO, M. 1987. A phylogenetic classification of Ophioglossaceae. Gard. Bull. Singapore 40:1–14.

KELLOGG, E. A. 1989. Comments on genomic genera in the Triticeae (Poaceae). Amer. J. Bot. 76: 796–805.

LES, D. H. 1994. Molecular systematics and taxonomy of lake cress (*Neobeckia aquatica*; Brassicaceae), an imperiled aquatic mustard. Aquatic Bot. 49:149–165.

MANHART, J. R. 1994. Phylogenetic analysis of green plant *rbcL* sequences. Molec. Phylog. Evol. 3:114–127.

NEALE, D. B., K. A. MARSHALL, and R. R. SEDEROFF. 1989. Chloroplast and mitochondrial DNA are paternally inherited in *Sequoia sempervirens* (D. Don) Endl. Proc. Natl. Acad. Sci. U.S.A. 86:9347–9349.

NEALE, D. B., and R. R. SEDEROFF. 1988. Inheritance and evolution of conifer organelle genomes. Pp. 251–264 *in* J. W. Hanover and D. E. Keathey, eds. *Genetic manipulation of woody plants.* Plenum Press, New York.

———. 1989. Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. Theor. Appl. Genet. 77:212–216.

NEI, M. 1972. Genetic distance between populations. Amer. Naturalist 106:283–292.

PARIS, C. A., F. S. WAGNER, and W. H. WAGNER. 1989. Cryptic species delimitation, and taxonomic practice in the homosporous ferns. Amer. Fern J. 79:46–54.

PRICE, R., and J. D. PALMER. 1993. Phylogenetic relationships of the Geraniaceae and Geraniales from *rbc*L sequence comparisons. Ann. Missouri Bot. Gard. 80:661–671.

RIESEBERG, L.H., S. BECKSTROM-STERNBERG, and K. DOAN. 1990. *Helianthus annuus* ssp. *texanus* has chlorplast DNA and nuclear ribosomal RNA genes of *Helianthus debilis* ssp. *cucumerifolius.* Proc. Natl. Acad. Sci. U.S.A. 87:593–597.

RIESEBERG, L. H., and S. BRUNSFELD. 1991. Molecular evidence and plant introgression. Pp. 151–176 *in* P. S. Soltis, D. E. Soltis, and J. J. Doyle, eds. *Molecular systematics of plants.* Chapman and Hall, New York.

RITLAND, K., and J. E. ECKENWALDER. 1992. Polymorphism, hybridization, and variable evolutionary rate in molecular phylogenies. Pp. 404–428 *in* P. S. Soltis, D. E. Soltis, and J. J. Doyle, eds. *Molecular systematics of plants.* Chapman and Hall, New York.

SAIKI, R. K., D. H. GELFAND, S. STOFFEL, S. J. SCHARF, R. HIGUICHI, G. T. HORN, K. M. MULLIS, and H. A. ERLICH. 1987. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491.

SAMBROOK, J., E. F. FRITSCH, and T. MANIATIS. 1989. *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SANGER, F. 1981. Determination of nucleotide sequences in DNA. Science 214:1205–1210.

SOLTIS, D. E., and P. S. SOLTIS. 1989. Allopolyploid speciation in *Tragopogon*: insights from chloroplast DNA. Amer. J. Bot. 76:1119–1124.

STEBBINS, G. L. 1950. *Variation and evolution in plants*. Columbia University Press, New York.

STEIN, D. B., and D. S. BARRINGTON. 1990. Recurring hybrid formation in a poplation of *Polystichum* ×*potteri*: Evidence from chloroplast DNA comparisons. Ann. Missouri Bot. Garden 77: 334–339.

SWOFFORD, D. L., and D. P. BEGLE. 1993. *PAUP: Phylogenetic analysis using parsimony. Version 3.1.1. User's manual.* Laboratory of Molecular Systematics, Smithsonian Institution, Washington, D.C.

TRYON, A. F., and B. LUGARDON. 1991. *Spores of the Pteridophyta: Surface, wall structure, and diversity based on electron microscope studies.* Springer-Verlag, New York.

TRYON, R. M., and A. F. TRYON. 1982. *Ferns and allied plants, with special reference to tropical America.* Springer-Verlag, New York.

VOGELSTEIN, B., and D. GILLESPIE. 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. U.S.A. 76:615–619.

WAGNER, F. S. 1993. Chromosomes of North American grapeferns and moonworts (Ophioglossaceae: *Botrychium*). Contr. Univ. Michigan Herb. 19:83–92.

WAGNER, W. H. 1980. A probable new hybrid grapefern, *Botrychium matricariifolium* × *simplex*, from central Michigan. Michigan Bot. 19:31–36.

———. 1983. Reticulistics: The recognition of hybrids and their role in cladistcs and classification. Adv. Cladist. 2:63–79.

———. 1991. New examples of the moonwort hybrid, *Botrychium matricariifolium* × *simplex*, (Ophioglossaceae). Canad. Field-Naturalist 105:91–94.

WAGNER, W. H., and F. S. WAGNER. 1981. New species of moonwort, *Botrychium* subg. *Botrychium* (Ophioglossaceae), from North America. Amer. Fern J. 71:20–30.

———. 1983a. Genus communities as a systematic tool in the study of New World *Botrychium* (Ophioglossaceae). Taxon 32:51–63.

———. 1983b. Two Moonworts of the Rocky Mountains; *Botrychium hesperium* and a new species formerly confused with it. Amer. Fern J. 73:53–62.

———. 1986. Three new species of moonworts (*Botrychium* subgenus *Botrychium*) endemic in western North America. Amer. Fern J. 76:33–47.

———. 1988. Detecting *Botrychium* hybrids in the Lake Superior region. Michigan. Bot. 27:75–80.

———. 1990a. Notes on the fan-leaflet group of moonworts in North America with descriptions of two new members. Amer. Fern J. 80:73–81.

———. 1990b. Moonworts (*Botrychium* subgenus *Botrychium*) of the Upper Great Lakes region and Canada, with descriptions of two new species. Contr. Univ. Michigan Herb. 17:313–325.

———. 1993. Ophioglossaceae C. Agardh: Adder's-tongue family. Pp. 85–106 *in* Flora of North America Editorial Committee, eds. *Flora of North America, north of Mexico. Vol. 2: Pteridophytes and gymnosperms.* Oxford University Press, New York.

———. 1994. Another widely disjunct, rare, and local North American moonwort (Ophioglossaceae: *Botrychium* subg. *Botrychium*). Amer. Fern J. 84:7–15.

WAGNER, W. H., F. S. WAGNER, and J. M. BEITEL. 1985. Evidence for interspecific hybridization in pteridophytes with subterranean mycoparasitic gametophytes. Proc. Roy. Soc. Edinburgh, 86B:273–281.

WAGNER, W. H., F. S. WAGNER, C. H. HAUFLER, and J. K. EMERSON. 1984. A new nothospecies of moonwort (Ophioglossaceae; *Botrychium*). Canad. J. Bot. 62:629–634.

WENDEL, J. F. 1989. New World tetraploid cottons contain Old World cytoplasm. Proc. Natl. Acad. Sci. U.S.A. 86:4132–4136.

WERTH, C. R. 1989. The use of isozyme data for inferring the ancestry of polyploid pteridophytes. Biochem. Syst. Ecol. 17:117–130.

WERTH, C. R., S. I. GUTTMAN, and W. H. ESHBAUGH. 1985. Recurring origins of allopolyploid species in *Asplenium*. Science 228:731–733.

WILLIAMS, S. E., V. A. ALBERT, and M. W. CHASE. 1994. Relationships of Droseraceae: A cladistic analysis of *rbcL* sequence and morphological data. Amer. J. Bot. 81:1027–1037.